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Antithrombotic and Antilesion Benefits without Hemorrhagic Risks by Inhibiting Tissue Factor Pathway

Key Words
Tissue factor
Vascular thrombosis
Vascular neointimal lesion formation
Angioplasty
Endarterectomy

Abstract
The effects of inhibiting tissue factor-dependent thrombus formation on vascular neointimal lesion formation have been evaluated by inhibiting tissue factor activity using intravenous injections of active-site inactivated recombinant factor VIIa (FVIIai) administered to baboons immediately prior to initiating bilateral femoral balloon artery angioplasty and surgical carotid endarterectomy. FVIIai abolished thrombus formation at sites of vascular injury and decreased vascular lesion formation by approximately 50 percent at 30 days. We conclude that thrombus formation at sites of vascular injury is predominately tissue factor-dependent and that transient inhibition of tissue factor activity prevents both vascular thrombosis and vascular lesion formation, which implies that transiently inhibiting tissue factor at the time of elective mechanical vascular procedures may be useful in reducing clinical restenosis.

Antithrombotic Efficacy for Aspirin- and Heparin-Resistant Thrombosis

Aspirin- and heparin-resistant thrombooclusive processes arising at sites of plaque rupture or mechanical interventional procedures used in the treatment of symptomatic atherosclerotic disease lead to heart attacks and strokes [1–3]. Accordingly, strategies for treating already established thrombus depend upon directly interrupting platelet recruitment by inhibiting thrombin, the principal agonist [4], or blocking platelet glycoprotein (GP)IIb/IIIa receptor-dependent cohesion, the final process in platelet recruitment [5, 6]. When preventing the formation of resistant arterial thrombus, additional approaches include reducing thrombin production by inhib-

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iting earlier reactions in the coagulation sequence [7], or impairment of vascular wall thrombogenicity.

Following denuding arterial injury, platelets spread and express functional GPIIb/IIIa receptors for adhesive molecules (primarily fibrinogen), resulting in cohesion with ambient platelets [5, 6]. Tissue factor exposed at sites of vascular damage triggers the activation of coagulation zymogens, leading to the rapid generation of thrombin [8, 9], which proteolytically activates platelets [4] and cleaves fibrinogen. Thrombin is the principal mediator of platelet activation at sites of arterial injury, leading to self-amplifying platelet recruitment. Activated platelets further amplify thrombin production by expressing phosphotidylserine-rich membrane surfaces that promote the assembly of coagulation enzyme-cofactor complexes [10]. Thrombin binds with fibrin in the forming thrombus; bound thrombin is susceptible to inactivation by direct inhibitors of thrombin, but resists the inhibitory effects of heparin or its derivatives [11]. Since bound thrombin mediates the recruitment of platelets into thrombus forming at sites of arterial injury, it constitutes a relevant target for developing more effective antithrombotic agents. Thrombin's structural domains, including the catalytic site and two flanking clusters of accessory binding domains, regulate thrombin's interactions with inhibitors [7].

Direct antithrombins include naturally occurring antithrombin peptides [12], synthetic competitive and irreversible antithrombin peptides [13], and chemical analogs that exhibit systemic activity after oral administration. Direct antithrombins inactivate both bound and soluble thrombin, and are therefore capable of interrupting platelet recruitment in aspirin- and heparin-resistant thrombotic processes developing at sites of arterial damage, albeit at doses substantially greater than that required to inactivate soluble thrombin [4, 11]. In experimental animals recombinant hirudin, cloned from the medicinal leech, *Hirudo medicinalis*, interrupts platelet-dependent thrombus formation at sites of mechanical arterial injury in pigs and nonhuman primates, although the doses required to inhibit platelet deposition also produce corresponding impairment in hemostatic function [14]. Hirudin is currently used in randomized clinical trials. Recently, the dosage levels have been decreased in these trials because of unexpectedly high rates of serious bleeding events [15, 16]. The tripeptide *D*-Phe-Pro-Arg inhibits thrombin-induced platelet aggregation and cleavage of fibrinogen in vitro and in vivo, demonstrating that inhibition of the catalytic site is alone sufficient for blocking thrombin's effects in vivo [13]. *D*-Phe-Pro-boroArg (*D*-FPRBOH) blocks thrombin's catalytic site through a transition-state mechanism, and exhibits antithrombotic effects in several different animal models of arterial thrombosis. Argatroban (MD-805) is relatively potent in vitro, but is relatively ineffective inhibiting platelet-dependent thrombus formation in vivo. Direct antithrombins interrupt platelet and fibrin deposition in a dose-dependent manner that is profound at the highest doses for blood exposed to all thrombogenic surfaces tested. Moreover, all of the direct antithrombins tested inhibit platelet hemostatic function in concert with their antithrombotic effects (table 1). Oral antithrombins with gradually improving bioavailability are currently being developed. Oral antithrombins should be useful in the outpatient management of acute deep venous thrombosis, substituting costly inpatient monitored heparin infusions, and as a potential alternative for warfarin in the chronic anticoagulant prevention of deep venous thrombosis and pulmonary embolism.

Table 1. Effects of inhibiting thrombin production on platelet deposition and surgical bleeding

Inhibitor	Bleeding time min	^{111}In-platelet deposition platelets × 10^9	Surgical bleeding ml
None	4.0 ± 1.2	2.7 ± 0.22	1.5 ± 0.7
Anti-GPIIb/IIIa MoAB (10 mg/kg)	>30	0.3 ± 0.1	>100
D-FPRCH$_2$Cl (150 nmol/kg/min)	>30	0.2 ± 0.1	>100
Hirudin (8 mg/kg/h)	19 ± 3	0.2 ± 0.1	79 ± 15
APC (5 mg/kg/h)	8.1 ± 1.5	0.1 ± 0.01	23 ± 7.5
TAP (0.8 mg/kg/h)	4.2 ± 0.7	0.2 ± 0.05	15 ± 7.0
FVIIai (1 mg/kg)	4.1 ± 0.5	0.2 ± 0.04	2 ± 0.05

D-FPRCH$_2$Cl = *D*-Phe-Pro-Arg chloromethylketone; TAP = tick anticoagulant peptide.

Platelet recruitment is also inhibited by anti-GPIIb/IIIa monoclonal antibodies, by naturally occurring peptides containing RGD or dodecapeptide sequences, and by synthetic competitive analogs. Inhibition of the platelet GPIIb/IIIa receptor by murine monoclonal antibodies has been shown in experimental models to prevent platelet thrombus formation after vascular injury and to significantly shorten the time to reperfusion using tissue-type plasminogen activator after thrombotic coronary occlusion [6, 17]. Achieving antithrombotic effects with murine monoclonal antibodies required doses that essentially eliminate GPIIb/IIIa receptor function on all circulating platelets, resulting in striking inhibition of platelet hemostatic function and substantial experimental bleeding at sites of tissue injury in nonhuman primates. Prolongation of the bleeding time in these studies correlates with surgical bleeding with experimental endarterectomy (table 1). In patients, humanized antiGPIIb/IIIa monoclonal antibodies reduce acute coronary complications following high-risk angioplasty [18]. This important 'proof-of-concept' study demonstrates the clinical usefulness of administering anti-GPIIb/IIIa monoclonal antibodies for aspirin- and heparin-resistant thrombotic complications of high risk angioplasty, although this benefit comes at the cost of significant abnormal bleeding. This positive outcome supports the rationale for developing small-molecule, orally active inhibitors of platelet GPIIb/IIIa-dependent recruitment [19]. A number of naturally occurring cysteine-rich single-chain polypeptides have been isolated from snake venoms that potently inhibit the binding of fibrinogen to GPIIb/IIIa receptors and abolish platelet aggregation, including trigramin, bitistatin, echistatin, kistrin, and applaggin [7]. In general, these biologic peptides effectively inhibit binding of all RGD-containing adhesive proteins with platelet GPIIb/IIIa receptors at affinities similar to monoclonal antibodies, although their effects are short-lived in vivo. A peptide isolated from the southwestern pygmy rattle snake *Sistrurus m. barbouri*, known as barbourin, specifically inhibits the binding of adhesive proteins with human platelet GPIIb/IIIa, as opposed to integrins on other cells. This specificity is a con-

sequence of substituting Arg by Lys, forming the unique recognition sequence KGD. Synthetic cyclic peptides containing the KGD sequence also potently inhibit the binding of human platelets with adhesive proteins but with greater specificity for platelet GPIIb/IIIa than the integrins on other cells [20]. The effects of this KGD-dependent specificity on the peptide's relative antithrombotic efficacy and hemostatic safety are currently being studied in the clinic.

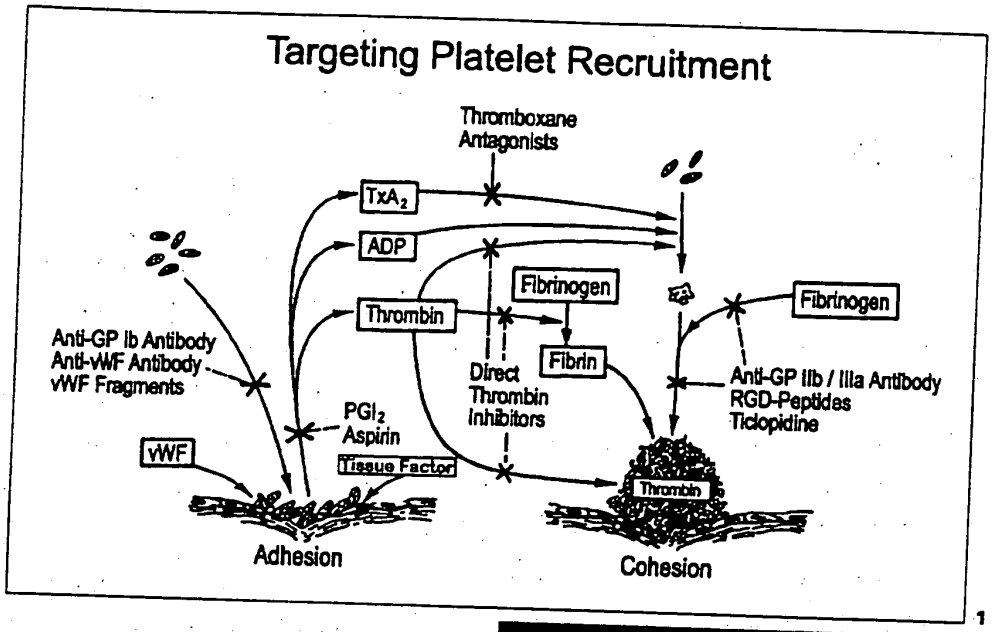
Ticlopidine is an effective oral antiplatelet agent that produces dose-dependent inhibition of GPIIb/IIIa-mediated platelet recruitment. It produces greater protection from vascular occlusive events than aspirin [21, 22] and is clinically useful [23–25] despite its troublesome side effects, which include neutropenia, diarrhea, hepatic dysfunction and skin rashes. Clopidogrel, an analog of ticlopidine producing fewer complicating adverse effects, is currently under development. Orally active synthetic small-molecule inhibitors of GPIIb/IIIa-dependent platelet recruitment are actively being developed for chronic oral therapy. The steep dose-response of these agents for inhibiting GPIIb/IIIa-dependent activity and antithrombotic effects, and the close association between antihemostatic versus antithrombotic effects lead to questions regarding the hemostatic safety of such chronic therapy.

Inhibition of Thrombin Production

In treating aspirin- and heparin-resistant thrombus that has already formed, platelet recruitment may be interrupted by inhibiting the agonist, bound thrombin, using direct antithrombins, or by blocking GPIIb/IIIa-dependent platelet cohesion with platelet receptor antagonist. However, both of these systemic therapies produce impairment in hemostatic function that is proportionate to their antithrombotic effects. An important strategy for reducing hemorrhagic risks of antithrombotic agents involves the inhibition of thrombin production.

Thrombus formation is prevented without compromising hemostatic plug formation by interrupting the production of thrombin using inhibitors of precursor proteases in the coagulation cascade (fig. 1, table 1). Because tissue factor in the arterial wall (or ruptured atherosclerotic plaque) initiates vascular thrombotic occlusion following angioplasty, stent placement, endarterectomy, or thrombolytic reperfusion, we have evaluated the relative antithrombotic and antihemostatic effects of inhibiting the tissue factor-dependent generation of thrombin. Vascular thrombosis at sites of carotid endarterectomy in nonhuman primates is prevented by bolus intravenous injections of inactivated factor VIIa (FVIIai), a competitive inhibitor of tissue factor (TF)-dependent activation of coagulation factor X, prior to restoring flow in the operated vessel (fig. 1, table 1). Importantly, the antithrombotic outcome is achieved without prolonging the bleeding time (a measure of platelet hemostatic function) or increasing surgical bleeding (table 1). FVIIai is produced by irreversibly interacting FVIIa with Glu-Gly-Arg chloromethylketone, and TF activity on endarterectomized arterial surfaces is abolished in vitro. The findings obtained in nonhuman primates suggest that inhibitors of TF-dependent thrombin production may be an effective and safe strategy for reducing the aspirin- and heparin-resistant thromboocclusive events complicating the use of interventional vascular procedures in the management of patients with symptomatic atherosclerotic disease.

Since FVIIai eliminates vascular thrombus formation at sites of mechanical vascular injury, we also investigated the effects of preventing vascular thrombosis on subsequent



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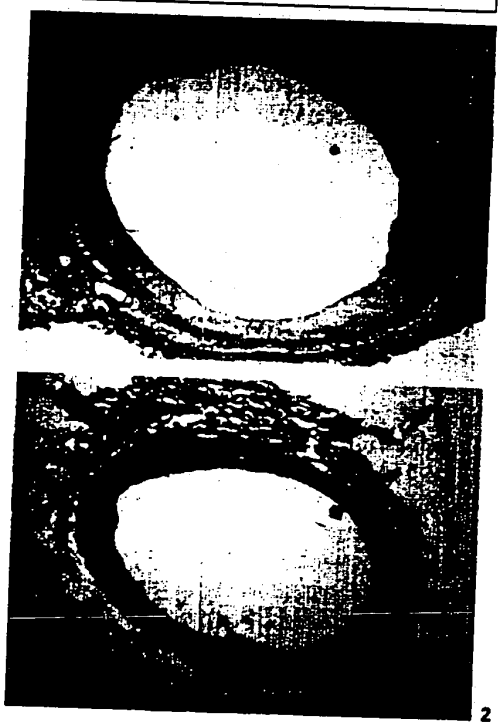

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Fig. 1. Central role of thrombin in platelet recruitment. Both soluble and bound thrombins activate platelets at concentrations less than that required to convert fibrinogen to fibrin. Platelet cohesion is mediated through adhesive protein bridging between GPIIb/IIIa receptors on adjacent activated platelets. Thrombin production on phosphatidylserine-rich activated platelet surfaces is amplified 300,000-fold via the tissue factor pathway.

Fig. 2. Antilesion effects of inhibiting tissue factor. **A** Cross-section of the lesion produced by surgical endarterectomy of a carotid artery. **B** Cross-section of a carotid artery 30 days after performing an endarterectomy in an animal given VIIai at the time of the procedure.

vascular lesion formation. When FVIIai was administered in antithrombotic doses for 1 week to 8 baboons undergoing surgical carotid endarterectomy and femoral artery balloon catheter angioplasty, vascular lesion formation was significantly decreased compared with 8 control animals (fig. 2). In these studies FVIIai was administered by bolus injections (5 mg/kg), followed by continuous intravenous infusion for 7 days (50 ng/kg/h). The steady-state plasma levels of FVIIai averaging 5 μg/ml inhibited TF activity in vitro, and decreased lesion formation by about 50% ($p = 0.02$) at 30 days. Thus, thrombus formation at sites of vascular injury is predominately TF-dependent, and transient inhibition of TF activity prevents both vascular thrombosis and vascular lesion formation. We conclude that thrombogenesis contributes to vascular lesion formation and that inhibiting TF transiently at the time of elective mechanical vascular procedures may be clinically useful in reducing thrombotic and restenotic complications.

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